A cellobiose-phosphorylase from Clostridium thermocellum

by Charles J Sih

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology
Montana State University
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This enzyme caused the phosphorolysis of cellobiose to glucose-1-phosphate and glucose. Its activity was measured by following the decrease in inorganic phosphate or the increase in glucose-1-phosphate. Phosphoglucomutase which destroys the glucose-1-phosphate was inactivated by freezing.

The phosphorylase was stable, being active over a wide range of temperature and pH. It was also specific in its action, resembling other disaccharide phosphorylases in its specificity and inhibition by the products of its action.

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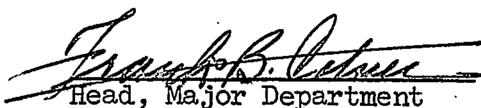
Approved:



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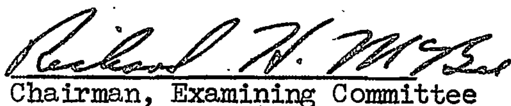

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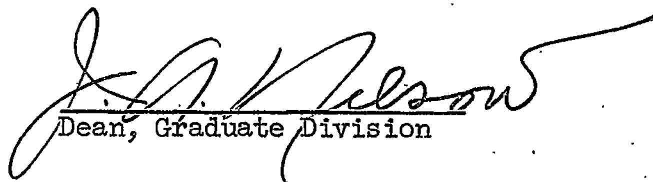

Dean, Graduate Division

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ABSTRACT

A cellobiose-phosphorylase was shown to be present in the cells of *Clostridium thermocellum* using cell-free enzyme preparations.

This enzyme caused the phosphorolysis of cellobiose to glucose-1-phosphate and glucose. Its activity was measured by following the decrease in inorganic phosphate or the increase in glucose-1-phosphate. Phosphoglucomutase which destroys the glucose-1-phosphate was inactivated by freezing.

The phosphorylase was stable, being active over a wide range of temperature and pH. It was also specific in its action, resembling other disaccharide phosphorylases in its specificity and inhibition by the products of its action.

The finding of this enzyme gives a partial answer to the question of how *Clostridium thermocellum* can use cellobiose as an energy source without being able to use exogenous glucose.

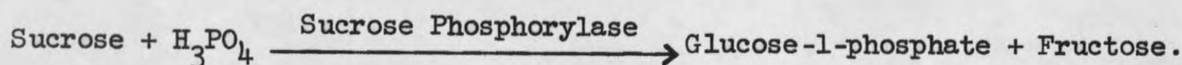
INTRODUCTION AND HISTORICAL REVIEW

Phosphorolysis is any process whereby inorganic phosphorus becomes esterified. In one type of phosphorolysis, phosphoric acid is substituted for water in a reaction similar to hydrolysis. This results in the formation of a phosphate ester instead of the usual hydrolytic product. Living systems which often require the expenditure of large amounts of energy to form sugar phosphates from monosaccharides may be able to obtain these same compounds from disaccharides and polysaccharides by phosphorolysis with a smaller outlay of energy. The phosphorolytic reactions are catalyzed by a variety of enzymes called phosphorylases which are widely distributed in animal and plant tissues where they play important roles in the synthesis and breakdown of many sugars and polysaccharides.

The first recognition of phosphorolysis occurred when Cori and Cori (1936) used dialyzed extracts of rabbit brain, heart, and liver to demonstrate that the glycolytic reactions were initiated by the formation of glucose-1-phosphate from glycogen and inorganic phosphate. Hanes (1940a and b) discovered a similar enzyme in extracts from peas and potatoes which would catalyze the phosphorolytic breakdown of starch and glycogen.

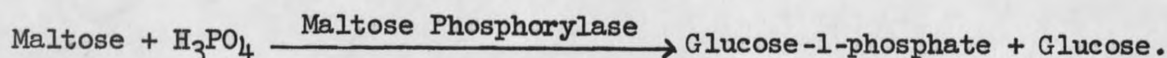
The phosphorolysis of disaccharides by bacteria was first discovered by Kagan, Latker, and Zfasman (1942) and Doudoroff, Kaplan, and Hassid (1943). The former group found a sucrose phosphorylase in

the cells of *Leuconostoc mesenteroides* and the latter recognized a similar enzyme in the cells of *Pseudomonas saccharophila*. The action of this enzyme may be represented as



$$\text{Sucrose} + H_3PO_4 \xrightarrow{\text{Sucrose Phosphorylase}} \text{Glucose-1-phosphate} + \text{Fructose.}$$

In 1952, Fitting and Scherp noted that maltose was broken down by phosphorolysis with an enzyme preparation from the cells of *Neisseria meningitidis*:



$$\text{Maltose} + H_3PO_4 \xrightarrow{\text{Maltose Phosphorylase}} \text{Glucose-1-phosphate} + \text{Glucose.}$$

The glucose-1-phosphate formed by these reactions is readily degraded by one of several energy yielding processes. The fate of the monosaccharide which is also formed by phosphorolysis is not as well understood. Some bacteria such as *Leuconostoc dextranicum* polymerize the sugar to form a dextran thus getting rid of a useless product. However, an unexplained situation is encountered when the living cells of these organisms will use disaccharides rapidly but not the monosaccharides, which are believed to be products of phosphorolysis. Such a situation exists when *Clostridium thermocellum* is grown on cellobiose. This species will use cellobiose but not glucose. Glucose is not found following growth on cellobiose, either as the free sugar or as a polymer.

Before the recognition of phosphorylases, the growth peculiarities of some of the cellulose-decomposing bacteria of the genus *Cytophaga*

were explained by Hutchinson and Clayton (1919) and Imesenecki and Solntseva in 1936 as inhibition of growth and cellulose decomposition by traces of glucose and other reducing sugars. They postulated that these were obligate cellulose-decomposing organisms, capable of utilizing cellulose directly without its first being hydrolyzed to sugars. Stainer (1942) found the same phenomenon if the glucose had been sterilized by heat. If, however, the glucose had been sterilized by filtration, the *Cytophaga* utilized it even more rapidly than cellulose. Therefore, he concluded that heat sterilization decomposed glucose to form toxic products which inhibited the growth of some cellulose-decomposing organisms. On the other hand, many workers have found the rapid utilization of disaccharides and practically non-utilization of monosaccharides. Doudoroff, Kaplan, and Hassid (1943) found that sucrose was rapidly utilized by the intact cells of *Pseudomonas saccharophila* whereas glucose was oxidized very slowly and fructose was not attacked. Yet fructose was the product both of the phosphorolysis and hydrolysis of sucrose by bacterial preparations. Doudoroff, *et al.*, (1949) also found a similar anomalous situation in the cells of a mutant strain of *Escherichia coli*. The living cells would metabolize maltose readily but not glucose. However, when bacterial-free enzyme preparations were used, glucose was formed in the breakdown of maltose.

Clostridium thermocellum is an anaerobic, thermophilic, cellulolytic organism. It was first obtained in pure culture by McBee (1948)

who reported that this organism attacked cellulose and cellobiose but not glucose, regardless of whether the glucose was sterilized by heat or filtration. The cellulose was first broken down to cellobiose by the enzyme cellulase and the latter was believed to be further broken down by the enzyme cellobiase to form two molecules of glucose. Since this organism did not utilize glucose in the culture medium, the hypothesis that cellobiose was hydrolyzed to glucose does not seem to be logical. The present study was therefore undertaken to determine whether unrecognized pathways of cellobiose utilization might occur in *Clostridium thermocellum*.

MATERIALS AND METHODS

Culture

Clostridium thermocellum, strain 157, was obtained from Dr. R. H. McBee who isolated it from soil in 1946. The characteristics of this organism were given by McBee (1954). Stock cultures were maintained in rolled tubes of cellulose agar using Hungate's (1950) anaerobic technique.

Culture Medium

The basic culture medium used throughout this work was composed of 0.075 per cent NaCl; 0.03 per cent $(NH_4)_2SO_4$; 0.03 per cent KH_2PO_4; 0.05 per cent K_2HPO_4; 0.01 per cent $MgSO_4$; 0.01 per cent $CaCl_2$; 0.5 per cent ball-milled cellulose or other carbohydrate; 0.1 per cent yeast extract; 0.02 per cent sodium thioglycollate; 0.5 per cent $NaHCO_3$ and a small amount of resazurin as an oxidation-reduction indicator. Because

$NaHCO_3$ is readily decomposed by heat, it was sterilized by filtration of a 10 per cent solution and added to the medium at a temperature below 60 C. For the rolled tubes the medium was solidified with 2 per cent agar. The cellulose was prepared in a 5 per cent aqueous suspension according to the directions of Hungate (1950).

Preparation of Large Cultures. Ten ml of a liquid cellulose medium was inoculated with a colony picked from a rolled tube of cellulose agar. After two or three days' incubation at 55 C this culture was used to inoculate 100 ml of medium. After three days at 55 C, this 100 ml served as an inoculum for two liters of medium which was in turn incubated for three days and used as the inoculum for a five gallon carboy of the medium. This was incubated at 55 C in a large water bath. Sterile medium in the carboy was kept reduced by bubbling carbon dioxide through it prior to inoculation, since it had been found that unless the resazurin in the medium was reduced to the colorless state, good growth could not be expected. This flushing with carbon dioxide was necessary for the first fifteen hours of incubation, after which time the organisms produced sufficient CO_2 and H_2 to keep the medium in a reduced state. A mechanical stirrer was then used to disperse the remaining cellulose throughout the medium, since it was found that fermentation proceeded more rapidly when the cellulose was kept suspended. When all the cellulose had disappeared (about 48 hours) the culture was neutralized with approximately 50 ml of glacial acetic acid to prevent an alkaline condition due to the loss of

CO_2. The cells were then harvested from the culture with a cream separator and used at once for making cell-free enzyme preparations. The cell yield was usually about 20 g wet weight from each five gallon culture.

Preparation of the Enzyme

The cells were broken employing McIlwain's (1948) method as recommended by Hayaishi and Stanier (1951). Grinding of the cells was done in a mortar at 0 to 5 C for ten minutes with twice their weight of alumina (Alcoa Chemicals alumina A-301). About 40 ml of M/35 sodium acetate-barbital buffer at pH 7.0 was added during the grinding to extract the soluble enzymes. The alumina and insoluble debris were removed by centrifugation. The speed of centrifugation was varied with the course of the experiments. The extracts obtained were used as the source of crude enzyme. The treatment of the extract also varied according to the experiment in which it was used.

Phosphorus Determination

The Fiske and Subbarow (1925) method of inorganic phosphate determination was used following precipitation of proteins with 100 per cent trichloroacetic acid. Heat-labile organic phosphates were determined by the same method following hydrolysis with 1 N hydrochloric acid for seven minutes in a boiling water bath. Inorganic phosphate is designated as Pi, the labile organic phosphate as $P_{\Delta 7}$.

Sugar Determination

Reducing sugars were determined by the Folin-Malmros (1929) method.

Paper Chromatography

Ascending paper chromatography was used to separate phosphate derivatives of sugars. The method of Hanes and Isherwood (1949) was modified, using tertiary-butanol-picric acid-water (80 ml, 4 g, 20 ml) and n-butanol-acetic acid-water (74 ml, 19 ml, 50 ml) (Benson et al., 1950) as solvents. Solutions containing unknown mixtures of sugar phosphates were spotted at 2.5 cm intervals along the starting line of a sheet of filter paper (Whatman No. 1 and 4) by means of a capillary pipette and allowed to air dry. The paper was then hung in a battery jar containing the solvent mixture so that the edge of the paper near the unknown spots dipped into the solvent. The jar was then covered with a glass plate and sealed with scotch tape to prevent evaporation of the solvent. After a period of at least 15 hours, the filter paper was removed from the jar, dried in an oven at 55 C, and developed to detect phosphate compounds. The papers were sprayed with a solution containing 5 ml of 50 per cent perchloric acid, 10 ml of 1 N HCl and 25 ml of 4 per cent ammonium molybdate per 100 ml. The sprayed papers were dried again at 55 C for a few minutes to remove excess water, then transferred to an 85 C oven, in which they were heated for seven minutes. This treatment even causes enough hydrolysis of such resistant esters as

3-phospho-glyceric acid and glucose-6-phosphate to render them detectable in minute amounts (Hanes and Isherwood 1949). The position of the phosphoric esters was visible at this stage, but to develop the color fully, the papers were allowed to regain moisture from the air and then placed in a jar containing H_2S for five minutes. The phosphoric esters then appeared as intensely blue spots against a buff background. The type of compound could be determined by its position on the chromatogram.

EXPERIMENTAL RESULTS

Growth Experiment

A preliminary examination of *Clostridium thermocellum* for the presence of a cellobiose phosphorylase was made by a simple growth experiment. A liter culture of the organism was grown in the basal medium containing cellobiose. After three days of incubation, not only was vigorous growth apparent, but also a decrease in inorganic phosphate was noted. However, when 0.5 per cent glucose was substituted for cellobiose in the above medium, no growth was obtained and no decrease in inorganic phosphate was noted (Table I). This confirmed the earlier observation that glucose was not utilized by this organism, and supported the theory that a phosphorylase might be present in this organism. Phosphate determinations after 30 days incubation showed an even larger uptake of phosphate in the cellobiose culture with no change in the glucose culture.

TABLE I.

Changes in inorganic phosphate of the culture medium during growth of *Clostridium thermocellum*.

	Substrate	
	Glucose	Cellobiose
Pi - mg		
Initial	2.81	2.86
After three days	2.81	2.64
After one month	2.81	2.61

Cell-Free Enzyme Experiments

In order to study more completely the phosphorylase which had been demonstrated in only a presumptive manner with a living culture, it was necessary to use enzyme preparations free of living cells. These were prepared by grinding the cells obtained from five gallon cultures in the manner already outlined.

Experiment 1. An enzyme preparation obtained from the grinding of 20 g of cell paste with 40 g of alumina was extracted with 40 ml of an M/35 sodium-acetate-barbital buffer, pH 7.0. The alumina and larger cell particles were removed by centrifuging for 20 minutes at a relative

centrifugal force of about 2000 G. The supernatant fluid was drawn off with a pipette. Such preparations yielded 25 to 30 ml of an opalescent crude enzyme mixture. A 6.15 ml portion of this fluid was placed in each of six 20 ml test tubes, two of which were then placed in a boiling water bath for 15-20 minutes to inactivate the enzymes. Sufficient 0.01 M phosphate solution was added to give an inorganic phosphate concentration of 0.16 mg/ml. Magnesium sulfate was added as an enzyme activator in a final concentration of 1.63 mg/ml, and 0.034 mg/ml of sodium fluoride was used to inhibit phosphatases which might interfere with the accumulation of organic phosphates. To one boiled control tube and to two of the other tubes was added 1.63 mg/ml of glucose. A similar quantity of cellobiose was added to the other three tubes. Inorganic phosphates and heat-labile phosphates were determined immediately after the addition of the sugars and also following a two-hour incubation at 37 C.

There was no significant phosphate change in the tubes to which glucose had been added (Table II). The addition of cellobiose, however, resulted in a decrease of the inorganic phosphate, indicating that phosphorolysis of the cellobiose had taken place. But there was no evidence that glucose-1-phosphate was a product of this reaction.

TABLE II

Changes in phosphate when a cell-free enzyme preparation acts on glucose and cellobiose. Results are those of duplicate experiments.

	Substrate					
	Glucose		Control	Cellobiose		Control
Pi - mg						
Initial	0.81	0.81	0.80	0.80	0.79	0.81
Final	0.92	0.95	0.83	0.64	0.58	0.82
	+0.11	+0.14*	+0.03	-0.15	-0.21	+0.01
$P_{\Delta 7}$ - mg						
Initial	0.00	0.00	0.01	0.04	0.02	0.01
Final	0.00	0.01	0.05	0.01	0.06	0.01
	0.00	+0.01	+0.04	-0.03	+0.04	0.00
P total - mg						
Initial	0.81	0.81	0.81	0.84	0.81	0.83
Final	0.92	0.96	0.88	0.66	0.64	0.83
	+0.11	+0.15	+0.07	-0.18	-0.17	0.00

*The increase in inorganic phosphate with glucose is probably due to the action of non-specific phosphatases on organic phosphates from the ground cells.

Experiment 2. A repetition of Experiment 1, using an enzyme preparation which had been frozen for several days gave similar results with respect to inorganic phosphate (Table III), and in addition showed an increase in the heat-labile phosphate.

The decrease in the inorganic phosphorus fraction was proportional to the increase in the 7 minute heat-labile organic phosphate fraction which contains those esters in which the phosphate radical is attached at the number 1 position on the sugar, such as glucose-1-phosphate. These are heat labile in dilute acid solutions as compared to the heat stable glucose-6-phosphate. Therefore, the 7-minute heat-labile organic phosphate fraction may be determined by subtracting the concentration of the inorganic phosphate fraction prior to hydrolysis from that following heating for 7 minutes at 100 C in 1 N HCl. This accumulation of heat-labile phosphate was encountered only when the enzyme preparation had been frozen for a few days. Otherwise there was a decrease in inorganic phosphorus but no corresponding increase in the 7-minute phosphorus fraction. This was attributed to the presence of phosphoglucomutase, an enzyme which converts glucose-1-phosphate (heat labile) to glucose-6-phosphate (heat stable). It appeared that the phosphoglucomutase was inactivated by freezing.

TABLE III

Changes in phosphate when a frozen enzyme preparation acted on glucose and cellobiose. Results are those of duplicate experiments.

	Substrate					
	Glucose		Control	Cellobiose		Control
Pi - mg						
Initial	1.07	1.06	0.99	1.07	1.07	1.05
Final	1.07	1.05	1.00	0.94	0.90	1.03
	0.00	-0.01	+0.01	-0.13	-0.17	-0.02
$P_{\Delta 7}$ - mg						
Initial	0.00	0.00	0.02	0.00	0.00	0.01
Final	0.01	0.01	0.02	0.11	0.13	0.00
	+0.01	+0.01	0.00	+0.11	+0.13	-0.01
P total						
Initial	1.07	1.06	1.01	1.07	1.07	1.06
Final	1.02	1.06	1.02	1.05	1.03	1.03
	-0.05	0.00	+0.01	-0.02	-0.04	-0.03

Experiment 3. The effect of freezing of the enzyme shown in Experiment 2 was confirmed on two additional enzyme preparations. The changes in phosphates in the presence of cellobiose were determined on a portion of the enzyme when it was prepared and again after having been frozen for one week (Table IV). In both cases, it was found that the phosphoglucomutase had been inactivated and that glucose-1-phosphate had accumulated.

Experiment 4. To determine whether the effect of freezing the enzyme preparation was truly due to an inactivation of phosphoglucomutase or possibly to some other reaction, an experiment using glucose-1-phosphate as the substrate was set up with fresh enzyme and with enzyme which had been frozen for one week. Freshly prepared enzyme converted nearly all of the glucose-1-phosphate to a heat stable compound, presumably glucose-6-phosphate, whereas the frozen enzyme had little, if any, effect on the concentration of heat-labile phosphate, indicating that the glucose-1-phosphate remained unaltered (Table V).

TABLE IV

Changes in phosphate when freshly prepared and frozen enzyme preparations were allowed to act on cellobiose. Results are those of duplicate experiments.

	Enzyme			
	Freshly prepared		Frozen for one week	
Pi - mg				
Initial	0.73	0.73	0.77	0.77
Final	0.51	0.49	0.59	0.59
	-0.22	-0.24	-0.18	-0.18
$P_{\Delta 7}$ - mg				
Initial	0.00	0.00	0.00	0.00
Final	0.00	0.00	0.10	0.11
	0.00	0.00	+0.10	+0.11
P total - mg				
Initial	0.73	0.73	0.77	0.77
Final	0.51	0.49	0.69	0.70
	-0.22	-0.24	-0.08	-0.07